A Peptide Derived from the Highly Conserved Protein GAPDH Is Involved in Tissue Protection by Different Antifungal Strategies and Epithelial Immunomodulation

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has an important role not only in glycolysis but also in nonmetabolic processes, including transcription activation and apoptosis. We report the isolation of a human GAPDH (hGAPDH) (2-32) fragment peptide from human placental tissue exhibiting antimicrobial activity. The peptide was internalized by cells of the pathogenic yeast *Candida albicans* and initiated a rapid apoptotic mechanism, leading to killing of the fungus. Killing was dose-dependent, with 10 μg ml (3.1 μM) and 100 μg ml hGAPDH (2-32) depolarizing 45% and 90% of the fungal cells in a population, respectively. Experimental *C. albicans* infection induced epithelial hGAPDH (2-32) expression. Addition of the peptide significantly reduced the tissue damage as compared with untreated experimental infection. Secreted aspartic proteinase (Sap) activity of *C. albicans* was inhibited by the fragment at higher concentrations, with a median effective dose of 160 mg l^{-1} (50 μM) for Sap1p and 200 mg l^{-1} (63 μM) for Sap2p, whereas Sap3 was not inhibited at all. Interestingly, hGAPDH (2-32) induced significant epithelial IL-8 and GM-CSF secretion and stimulated Toll-like receptor 4 expression at low concentrations independently of the presence of *C. albicans*, without any toxic mucosal effects. In the future, the combination of different antifungal strategies, e.g., a conventional fungicidal with immunomodulatory effects and the inhibition of fungal virulence factors, might be a promising treatment option.

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INTRODUCTION

Owing to the increased number of immunosuppressed patients resulting from AIDS, organ transplants, and drug addiction, the frequency of fungal infections with the opportunistic yeast *Candida albicans* has risen (Leroy *et al.*, 2009). These infections, especially oropharyngeal candidiasis, are widely treated with fluconazole. The long-term treatment with azoles has led to the emergence of azole-resistant strains of *C. albicans* (Fera *et al.*, 2009). Azoles inhibit the key enzyme of ergosterol biosynthesis, cytochrome P-450-dependent lanosterol-14α-demethylase. Known mechanisms of azole resistance are the decreased availability of lanosterol-14α-demethylase and efflux pumps (Peman *et al.*, 2009; Sanglard *et al.*, 2009).

Another therapeutic option is to affect fungal virulence factors, such as secreted hydrolytic enzymes, yeast-to-hyphal transition, adhesion factors, phenotypic switching, thigmotropism, and molecular mimicry. During the last decade, secreted aspartic proteinase (Sap) activity has been characterized as one of the major virulence factors of *C. albicans* (Schaller *et al.*, 2005). Ten *SAP* genes have been identified in *C. albicans.* The corresponding proteinases are crucial for distinct steps in pathogenesis, such as adhesion and penetration. The interest in Sap inhibitors started with the treatment of AIDS patients with highly active antiretroviral therapy, a combination of HIV aspartic proteinase and

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Abbreviations: C. albicans, Candida albicans; hBD-3, human β-defensin-3; hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LDH, lactase dehydrogenase; PBS, phosphate-buffered saline; RHE, reconstituted human oral epithelium; Sap, secreted aspartic proteinase; TLR, Toll-like receptor

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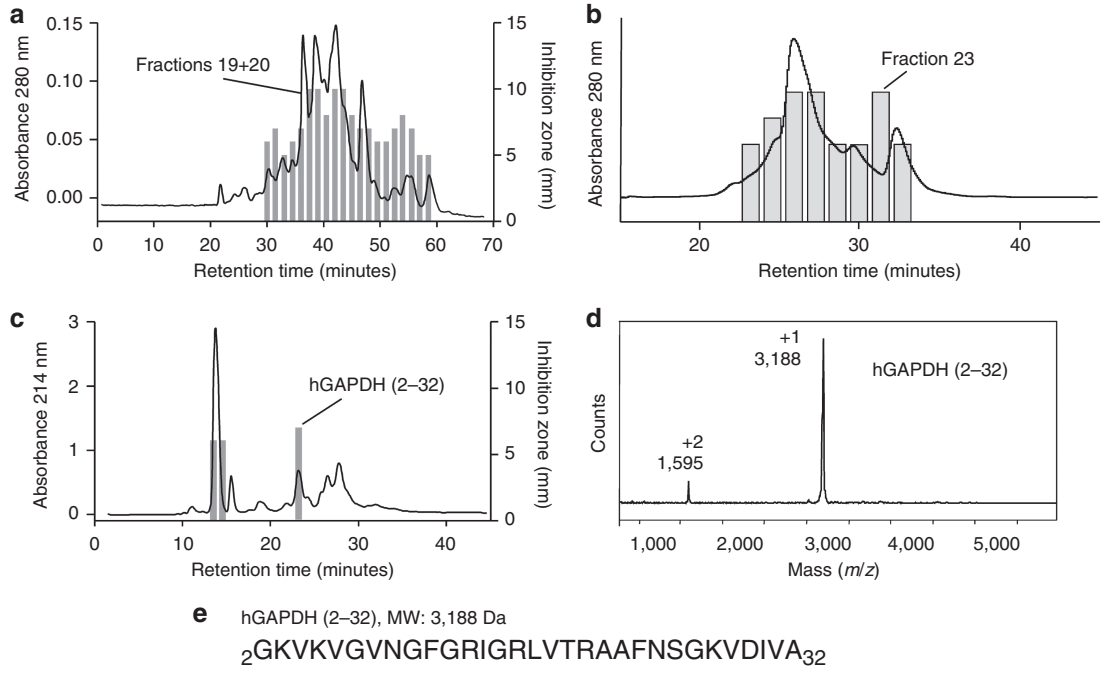

Figure 1. Purification of the antimicrobial peptide human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (2-32) from human placental tissue. Each purification step was monitored by radial diffusion assay for detection of antimicrobial activity. (**a**) The bars show the diameters of inhibition zones indicating the antimicrobial activity against *E. coli*. Fractions 19 and 20, corresponding to the maximum growth inhibition, were selected for further purification. (**b**) Fractions 19 and 20 were pooled and fractionated by reversed-phase (RP) chromatography. Final purification of the antimicrobial peptide was performed, (**b**) separating fraction 23 (**c**) using a strong cation-exchange column. (**d**) Matrix-assisted laser desorption/ionization mass spectrometry analysis of the purified peptide revealed a molecular mass of 3,188 Da. Sequence analysis led to the identification of an N-terminal fragment of GAPDH. (**e**) Amino-acid sequence is shown in the single-letter code. MW, molecular weight.

reverse transcriptase inhibitors. Some of the clinically used HIV proteinase inhibitors, e.g., saquinavir and indinavir, also have the ability to inhibit Sap activity and therefore may prevent fungal infections or reduce their severity (Korting *et al.*, 1999). This finding led to an enhanced effort of focusing research on specific Sap inhibitors by solving the X-ray crystal structures of these proteinases and by the development of Sap-specific inhibitors (Borelli *et al.*, 2008; Braga-Silva and Santos, 2011). Most HIV aspartic proteinase inhibitors and Sap inhibitors are short peptide-like ligands mimicking substrates for HIV proteinase (Gauwerky *et al.*, 2009). Moreover, it has been shown that lysozyme (muramidase), an antimicrobial peptide effective against a wide range of bacteria, not only inhibited the growth of *C. albicans* but also decreased the secretion of the isoenzyme Sap2, which is the predominant secreted isoenzyme (Wu *et al.*, 1999).

In the present investigation, we examined the effect of an antimicrobial peptide, purified from human placental tissue, on the growth of *C. albicans*, and on Sap activity. In addition, we analyzed the protective and immunomodulatory effects during epithelial infection.

RESULTS

Purification of hGAPDH (2-32) from human placental tissue

A peptide library orginating from human placental tissue was initially screened for growth inhibition against *E. coli* using a radial diffusion assay. Antimicrobial activity was detected in several HPLC fractions of pH pool 4. Of this pool, the fractions 19 and 20, which exhibited the highest growth-inhibitory activity, were selected for further purification (Figure 1a). To isolate the active compounds, two subsequent HPLC steps were carried out, tracking the maximum antimicrobial activity within the resulting fractions. The antimicrobial active fractions were loaded onto a reversed-phase column. Elution of bound material was carried out by linearly increasing the amount of solvent B. Growth-inhibitory activity was eluted over a broad range, and antibacterial fractions were selected for further purification. As it is known that most antimicrobial peptides are of a cationic nature, a strong cation-exchange column was used for final purification. The resulting three HPLC fractions showing the strongest antimicrobial activity (Figure 1b) were desalted and subsequently tested for further analyses (Figure 1c). Analysis of HPLC fraction 23 by capillary zone electrophoresis revealed a compound of high purity (data not shown).

The molecular mass of the purified peptide was determined to be 3,188 Da measured by matrix-assisted laser desorption/ionization mass spectrometry (Figure 1d).

Edman degradation yielded the following peptide sequence of 31 amino-acid residues: GKVKVGVNGFGRIGRL VTRAAFNSGKVDIVA. Comparison of the amino-acid sequence obtained with the SwissProt Database showed 100% identity with the N-terminal fragment of the human protein glyceraldehyde-3-phosphate dehydrogenase (Figure 1e).

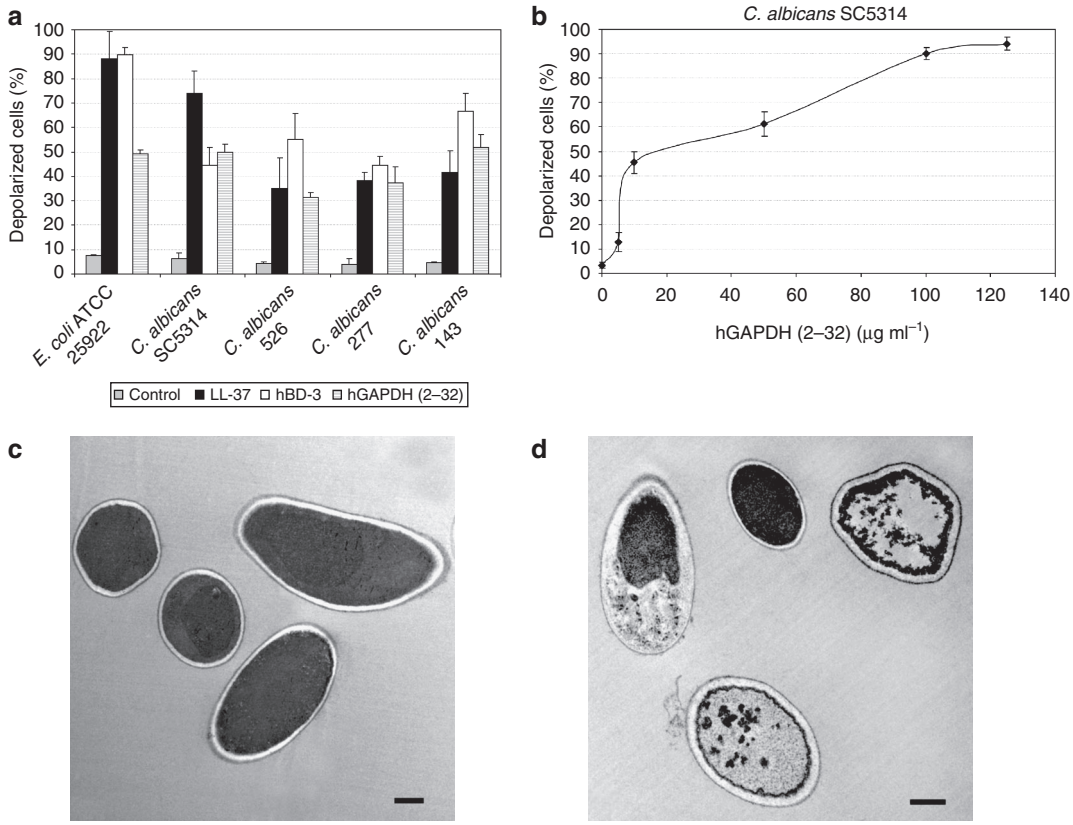

Figure 2. Antimicrobial killing assays and electron microscopy. (**a**) Flow cytometric antimicrobial killing assay of *E. coli* and *Candida albicans (C. albicans)* incubated with 10 μg ml^{-1} human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (2-32), LL-37, or human β-defensin-3 (hBD-3). Dose-dependent effect of hGAPDH (2-32). Suspensions of *C. albicans* were incubated with hGAPDH (2-32) for 90 minutes. (**b**) The antimicrobial activity is shown as percentage of depolarized microorganisms. The data are means of one representative experiment in triplicate. Electron microscopy of *C. albicans* SC5314 cells grown (**c**) without and (**d**) with 125 μg ml^{-1} hGAPDH (2-32) for 24 hours. (**c**) Cells grown without hGAPDH (2-32) with a regular morphology. (**d**) *C. albicans* grown under the influence of hGAPDH (2-32) shows enlargement of cytoplasmic vacuoles and disorganization of the internal organelles. Bar = 500 nm.

Functional characterization of the synthetic antimicrobial peptide

Antimicrobial activity. To analyze the identity and biological properties of the isolated peptide, human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (2-32) was chemically synthesized. To specify the spectrum of activity of the peptide, its antimicrobial activity was determined by a flow cytometric antimicrobial killing assay (Nuding *et al.*, 2006). This assay demonstrated a potent antibacterial activity of this peptide against *E. coli* (Figure 2a) and confirmed the results of the radial diffusion assay, which was originally used to screen the HPLC fractions for growth-inhibitory activity against this Gram-negative bacterium (Figure 1c). Two of the four tested *C. albicans* strains were similarly highly susceptible to the hGAPDH (2-32) fragment, with 10 μg ml^{-1} depolarizing ~50% of the fungi in a population, whereas two other strains showed a slightly lower sensitivity (Figure 2a). We compared the activity spectrum of our peptide with that of LL-37 and human β-defensin-3 (hBD-3). The antifungal activities of all three peptides against *C. albicans* were not significantly different, whereas hGAPDH (2-32) was less active against *E. coli*. The antifungal effect of h(GAPDH) 2-32 was dose dependent, as shown for *C. albicans* SC5314 (Figure 2b).

The antimicrobial activity was also confirmed by the broth microdilution method (minimal inhibitory concentration 100%) against several bacterial strains and *C. albicans*. The identified peptide exhibited a broad spectrum of activity, inhibiting the growth of Gram-negative bacteria (*E. coli* BL21, *Pseudomonas aeruginosa (P. aeruginosa)* PAO, *P. aeruginosa* clinical isolate) and *C. albicans* in micromolar concentrations (data not shown).

Ultrastructural changes of C. albicans morphology induced by hGAPDH (2-32). Whereas untreated *C. albicans* SC5314 cells were in a uniform physiological state (Figure 2c), incubation of yeasts with hGAPDH (2-32) in a concentration of 125 μg ml^{-1} for 24 hours resulted in distinct changes of the cell wall, plasma membrane, and the cytoplasm (Figure 2d). Morphological alterations included enlargement of the fungal cytoplasmic vacuoles, disorganization of the internal organelles, and the appearance of yeasts with an empty cytoplasm resembling necrotic ghost cells.

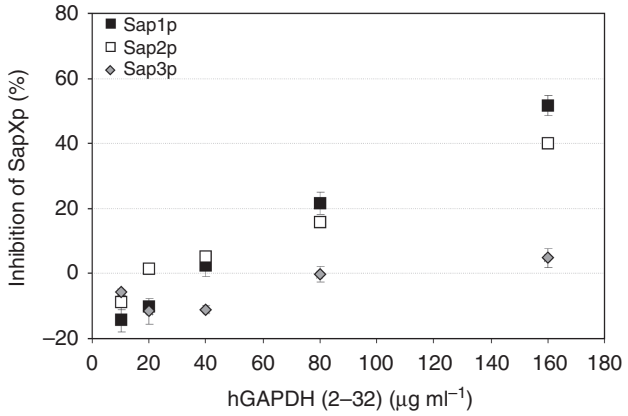

Figure 3. Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (2-32) inhibits the activity of secreted aspartic proteinases (Saps), virulence attributes of *Candida albicans* (*C. albicans*). Specific inhibition of Sap1-3 showed an ED_{50} (median effective dose) of 160 μg ml^{-1} (50 μM) for Sap1p and 200 μg ml^{-1} (63 μM) for Sap2p, while Sap3p was not inhibited. Error bars indicate range of duplicates. Each duplicate consists of three background-normalized measures.

Inhibition of Sap activity. Addressing the question of whether hGAPDH (2-32) has an inhibitory activity against *C. albicans* Sap, we tested inhibition of the recombinant proteins Sap1, 2, and 3. The Sap-specific test showed an ED_{50} (median effective dose) of 160 μg ml^{-1} (50 μM) for Sap1p and 200 μg ml^{-1} (63 μM) for Sap2p, whereas Sap3 was not inhibited (Figure 3).

Experimental C. albicans infection. We used epithelial monolayers of the TR146 cell line to analyze the antimicrobial effect of hGAPDH (2-32) during infection. LDH values 12 and 24 hours after infection with *C. albicans* SC5314 were significantly decreased even in the presence of the lowest hGAPDH (2-32) concentration of 5 μg ml^{-1} as compared with untreated controls (Figure 4a). The protective effect of hGAPDH (2-32) was similar to that of LL-37. Treatment of uninfected epithelial cells with hGAPDH (2-32) also demonstrated that this peptide has low toxicity on mammalian cells (Figure 4a). Analyses of standard LDH samples in the absence and presence of 5 and 125 μg ml^{-1} hGAPDH (2-32) excluded inhibition of the LDH enzymatic assay by the antimicrobial peptide. We also demonstrated that LDH is exclusively secreted by the epithelial cells and not by *C. albicans* cells (data not shown).

Histological examination of reconstituted human oral epithelium (RHE), which consists of differentiated multilayers of the TR146 cell line taken 18 hours after infection with *C. albicans* SC5314, demonstrated clusters of fungal cells on the superficial keratinocytes and prominent lesions with edema and vacuolization of the keratinocytes and enlarged intercellular spaces as a sign of spongiosis. *C. albicans* effectively invaded all keratinocyte layers of the oral epithelium, affecting the tissue with a high number of microorganisms (Figure 4b). In contrast, histological examination of infected samples treated with 125 μg ml^{-1} hGAPDH (2-32) demonstrated far less marked morphological alterations. Spongiosis and invasion of keratinocytes by yeast cells were inhibited by hGAPDH (2-32), only the formation of mild edema in the uppermost keratinocyte layers resulted from *Candida* infection. In accordance with the minor tissue lesions observed, the number of yeast cells on the mucosal surface was decreased (Figure 4c).

We used an anti-hGAPDH (2-32) antiserum raised in rabbits to carry out immunofluorescence in our RHE model by confocal laser microspcopy. The specificity of the generated antibody against hGAPDH (2-32) was verified by ELISA, demonstrating a signal against the hGAPDH (2-32) peptide but no signal against the hGAPDH protein. The specificity of our antibody was further confirmed in our RHE experiments by staining of the peptide only in the hGAPDH (2-32)-treated (Figure 4e and g) and/or *C. albicans*–infected samples (Figure 4f and g) but not in the untreated and uninfected RHE (Figure 4d). Confocal laser scanning microscopy showed increased endogenous expression of hGAPDH (2-32) during RHE infection (Figure 4f), whereas no evidence for staining was seen in the uninfected phosphate-buffered saline (PBS) control (Figure 4d). In addition, we were able to demonstrate a strong affinity of the external but also of the endogenous peptide to *C. albicans* and internalization by the fungal cells (Figure 4f and g higher magnifications).

Apoptosis and necrosis of C. albicans in the presence of hGAPDH (2-32). Several reports about apoptotic cell death induced by antimicrobial peptides prompted us to investigate whether an apoptosis-like process occurs in hGAPDH (2-32)-treated *C. albicans* cells. Cells dying under these conditions display several markers characteristic of apoptosis. These include the rapid exposure of phosphatidylserine, detectable at the outer cell membrane by annexin V–FITC staining. In our assay, apoptotic and necrotic cells were distinguished by double staining for annexin V-FITC (green) and propidium iodide, which is a membrane-impermeant DNA fluorescent stain (Figure 5). Cells exposed to 5 μg ml^{-1} hGAPDH (2-32) for 1 hour (Figure 5c and e) showed peripheral fluorescence in 31% of the protoplasts. *C. albicans* cells treated for a longer time with this concentration or at higher doses of these compounds showed cellular changes characteristic of necrosis (Figure 2d). Annexin V–FITC-stained cells were observed only rarely in control assays performed without peptide (Figure 5a and b).

Immunomodulatory activity of hGAPDH (2-32). We investigated the role of hGAPDH (2-32) in initiating an epithelial chemokine response. Protein secretion and gene expression were quantified in the presence and absence of *C. albicans* SC5314 and 5 μg ml^{-1} hGAPDH (2-32) by use of ELISA and real-time reverse transcriptase–PCR. In response to *C. albicans,* epithelial cells secreted significantly increased amounts of IL-8 and GM-CSF (Figure 6a), which is in line with our previous studies (Weindl *et al.*, 2007). Surprisingly, hGAPDH (2-32) also induced increased secretion of GM-CSF and IL-8 of uninfected epithelial. IL-8 was increased even after *C. albicans* infection (Figure 6a and b). A similar stimulation pattern was also observed for epithelial Toll-like receptor 4 (TLR4) mRNA expression (Figure 6c).

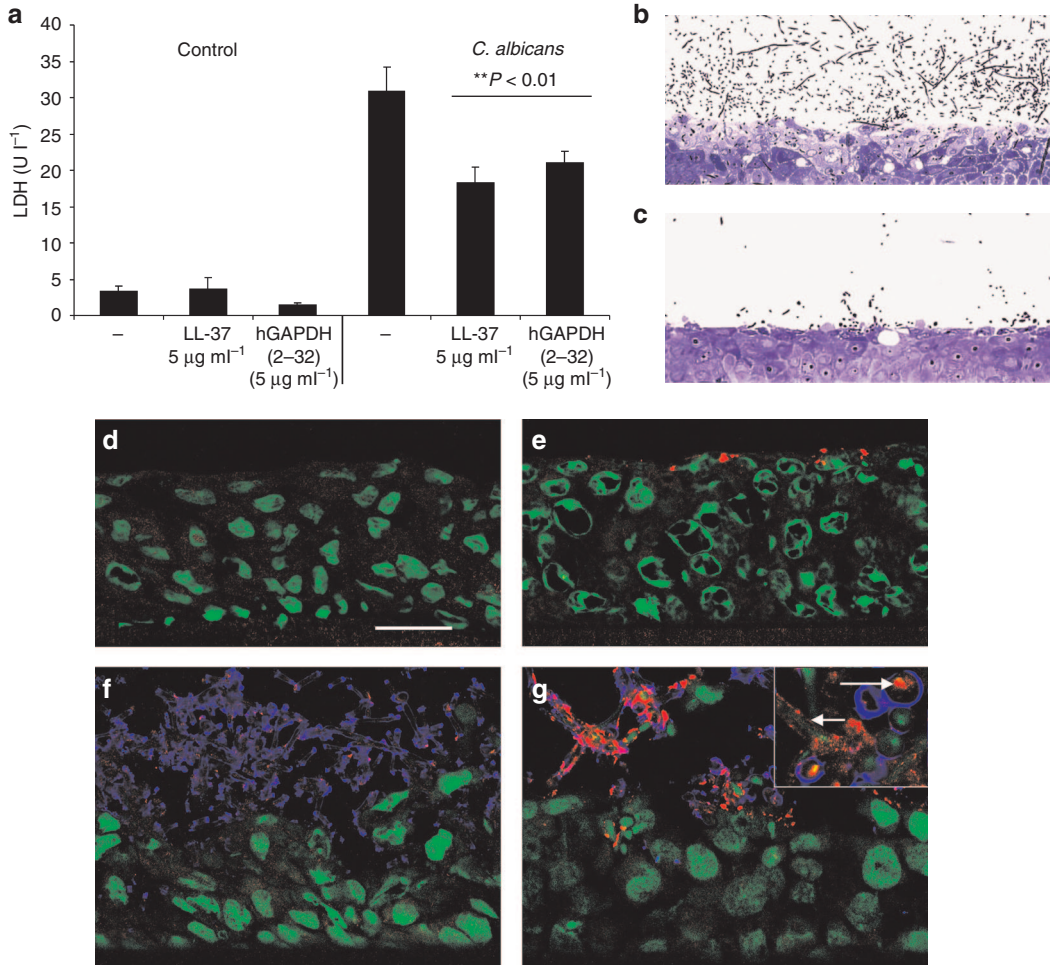

Figure 4. Experimental *Candida albicans (C. albicans)* infection. Release of lactase dehydrogenase (LDH) by monolayer epithelial cells 12 hours after infection (or not) with *C. albicans* in the presence and absence of 5 µg ml^{-1} LL-37 or human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (2-32). Multiplicity of infection: 0.1. (**a**) Epithelial cells were preincubated with the peptide for 1 hour. Statistical significance was determined using the two-tailed paired Student's *t* test ($n = 6$). Light micrographs of reconstituted human oral epithelium (RHE) 18 hours after infection with *C. albicans* SC5314 in the absence and presence of hGAPDH (2-32). (**b**) Invasion by *C. albicans* of all epithelial layers, with extensive edema and vacuolization in the absence of hGAPDH (2-32). Strongly reduced virulence phenotype resulting in a protective effect in the presence of hGAPDH (2-32). (**c**) Decreased number of *C. albicans* cells. Confocal laser microscopy of oral RHE after 12 hours in the presence and absence of *C. albicans* and 5 µg ml^{-1} hGAPDH (2-32) (cell nuclei, green; hGAPDH (2-32), red; *C. albicans*, blue). (**d**) No evidence for hGAPDH (2-32) in the uninfected and untreated oral RHE. (**e**) The presence of hGAPDH (2-32) on the superficial layers of the uninfected RHE after external addition. (**f**) Increased expression in the *C. albicans*-infected but untreated oral RHE. (**f**, **g**) Strong affinity of the peptide to the *C. albicans* cells after external addition of hGAPDH (2-32). Higher-magnification image demonstrating direct contact of the peptide with *C. albicans* cells (short arrow) and internalization by the fungal cells (long arrow). Bar = 30 µm. **$P < 0.01$, IL-37 and hGAPDH.

Extensive studies ruled out any effects of possible contamination with lipopolysaccharide (<100 pg ml^{-1}). These data thus indicate that hGAPDH (2-32) possesses immune stimulatory activity that might contribute to the protective effect.

DISCUSSION

With the aim of identifying naturally occurring antimicrobial active peptides in humans, we established a peptide library from human placental tissue as a known source for low-molecular-mass antimicrobial components. Purification procedures led to the isolation of a GAPDH-derived peptide with antibacterial and anticandidal properties. Although its glycolytic function, the conversion of D-glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, is well known, recent evidence suggests that this highly versatile molecule has several diverse roles in living systems. Mammalian GAPDH is involved in membrane fusion (Morero *et al.*, 1985), vesicle transport (Tisdale, 2001), microtubule bundling (Sirover, 1999), phosphotransferase activity, nuclear RNA export (Singh and Green, 1993), prostate cancer progression, programmed cell death, DNA replication, and DNA repair (Sirover, 1999, 2011). This came as a surprise to researchers, but it makes evolutionary sense to reuse and adapt an existing protein instead of evolving a novel protein from scratch. It also makes sense that a GAPDH fragment acts as an antimicrobial peptide, because GAPDH is widely expressed in a multitude of tissues (Barber *et al.*, 2005) and is found not only intracellularly but also extracellularly (Yamaji *et al.*, 2005). Cell injury by invading pathogens might

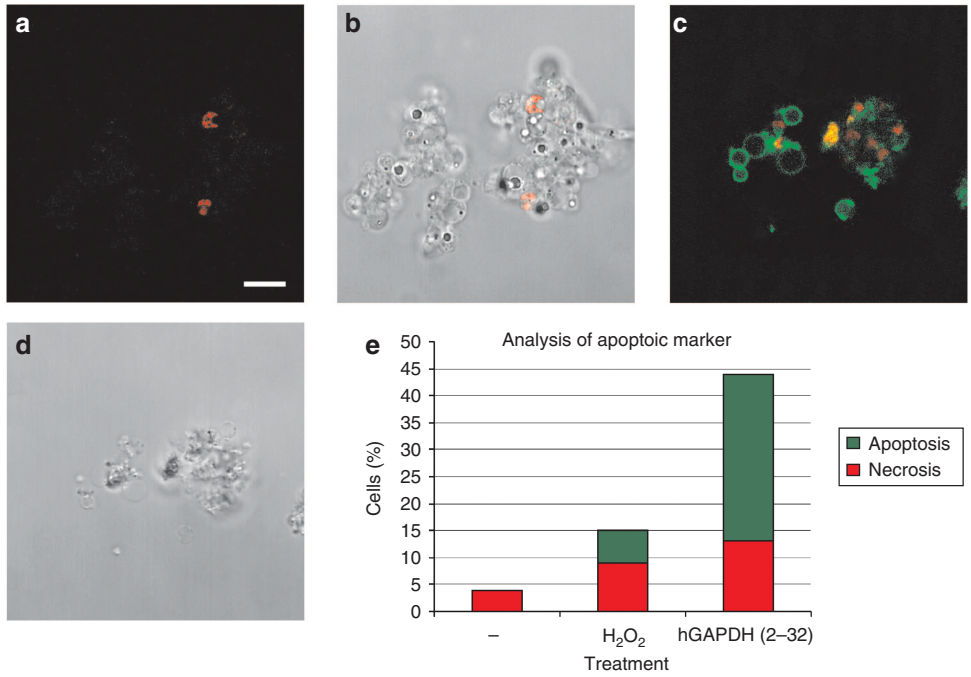

Figure 5. Apoptotic marker induced by 5 μg ml^{-1} human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (2-32) on *Candida albicans (C. albicans)* cells. Representative micrographs showing cells stained with FITC-annexin V (green) and propidium iodide (PI, red) to detect apoptosis (phosphatidylserine externalization) and necrosis, respectively. The cells were (**a**, **b**) untreated or (**c**, **d**) previously treated with hGAPDH (2-32) for 1 hour. **b** and **d** are phase-contrast micrographs. **a** and **b** show annexin and PI staining. (**e**) Percentage of 300 fungal cells that are classified as apoptotic (annexin (+) PI(−); green bars) and necrotic (annexin(+/−) PI(+); red bars) after treatment with hGAPDH (2-32) and H_2O_2. Bar = 10 μm.

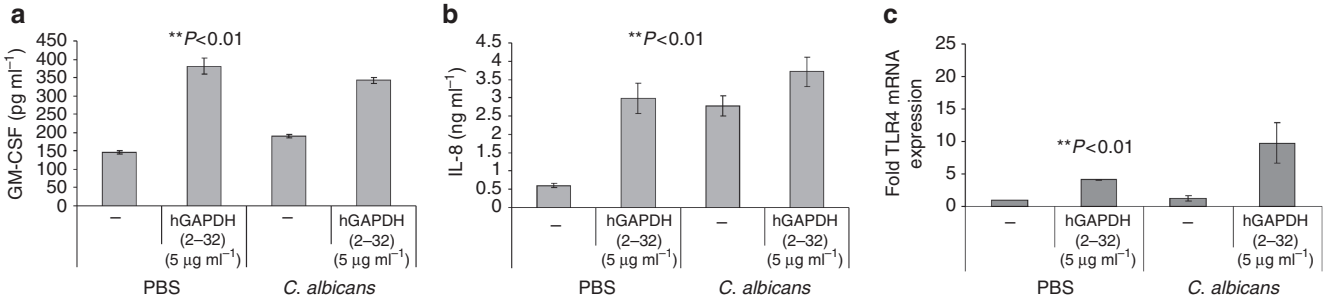

Figure 6. Expression of cytokines and Toll-like receptor 4 (TLR4) by human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (2-32). (**a**) GM-CSF, (**b**) IL-8 secretion, and (**c**) TLR expression of epithelial cells 12 hours after infection (or not) with *Candida albicans (C. albicans)* SC5314 in the presence and absence of 5 μg ml^{-1} hGAPDH (2-32) ($n = 6$). Data are expressed as means ± SD from duplicate assays of three different experiments. Statistical significance was determined using the two-tailed paired Student's *t* test. A *P*-value of 0.01 or less was considered significant ($n = 6$). PBS, phosphate-buffered saline.

even enlarge this extracellular fraction, and proteolytic degradation of GAPDH by the pathogen or the host might lead to the generation of smaller peptides with antimicrobial activity. Most recently, a role of microbial GAPDH in virulence has been postulated (Egea *et al.*, 2007; Dumke *et al.*, 2011). Moreover, evidence has been shown that GAPDH is relevant for the functioning of cationic host defense peptides as a mononuclear cell receptor for human cathelicidin LL-37 and immunomodulatory IDR-1 (Mookherjee *et al.*, 2009).

The isolated peptide exhibits antimicrobial activity against *E. coli* and *C. albicans* in micromolar concentrations. It is very difficult to compare the antifungal activity of hGAPDH (2-32) in our experiments with those of the peptides described earlier in the literature, because it is well known that their activity is highly reliant on the conditions used to test antifungal activity. We therefore used the depolarization assay described in this paper to compare the antifungal activity of hGAPDH (2-32) with those of LL-37 and hBD3. The antifungal activities of all three peptides against *C. albicans* were not significantly different, whereas hGAPDH (2-32) was less active against *E. coli*. The relevance of our peptide as an antifungal peptide is confirmed by the observation that antifungal activity was also demonstrated in different media by the radial diffusion test and microdilution broth method.

In addition, we found a protective effect during *C. albicans* infection of epithelial monolayers and multilayers comparable to the effect of LL-37 but independently from the culture media conditions.

The morphological changes observed in hGAPDH (2-32)-treated *C. albicans* cells are related mainly to cytoplasm and the organelles, suggesting internalization of the peptide. We were able to confirm cellular uptake after binding of the peptide to the cell wall of the fungi by immunofluorescence microscopy. The induction of apoptosis by hGAPDH (2-32) was characterized as a major killing mechanism by the exposure of phosphatidylserine from the inner to outer surface of the membrane as a representative hallmark of early-stage apoptosis.

The ongoing spread of antimycotic resistance is seriously undermining the present alternatives for therapeutic intervention against human candidiasis. Therefore, it becomes important to search and develop targets for antimicrobial therapy. Over the past few decades, the search for drugs and drug targets has prompted an interest in antimicrobial peptides. These are important components of the innate immune system, used by the host to protect itself from different types of pathogenic microorganisms. Besides their direct effects on microbial cells, there is a growing body of evidence that mammalian antimicrobial peptides directly influence host cells, thereby inducing several mechanisms of the inflammatory processes and an immune response to the invading pathogens (Schittek *et al.*, 2008). In this study, we suggest a role of antimicrobial peptides during host–pathogen interaction by inhibition of aspartic proteinases secreted by *C. albicans*. The production of these Sap by *C. albicans* is a putative virulence attribute of these opportunistic yeasts (Zhu and Filler, 2010). Preincubation of epithelial cells with the proteinase inhibitor pepstatin A before *C. albicans* infection significantly reduced invasion compared with untreated cells (Dalle *et al.*, 2010). Experiments using triple mutants, lacking Sap1-3, confirmed the role of these isoenzymes for induced endocytosis (Dalle *et al.*, 2010). An ED_{50} of 160 $\mu g\,ml^{-1}$ or 50 μM hGAPDH (2-32) for Sap1p and 200 $\mu g\,ml^{-1}$ or 63 μM for Sap2p suggests that inhibition of these important virulence factors only partially contributes to epithelial protection in our experiments. A more significant role of this peptide as a Sap inhibitor *in vivo* cannot be excluded, as GAPDH is widely expressed and abundantly present in a multitude of tissues, especially after epithelial injury during mucosal infection.

The outlook for the effectiveness of therapeutic approaches combining inhibitory effects on the growth and against virulence factors of *C. albicans* appears quite favorable. Lysozyme is an antimicrobial protein ubiquitous in human mucosal secretions such as saliva and effective against a wide range of bacteria. In a previous study, it has been shown that lysozyme has a bimodal action on *C. albicans*, killing the organism at higher concentrations and decreasing the production of Sap at lower concentrations (Wu *et al.*, 1999).

The peptide hGAPDH (2-32) is secreted by oral epithelial cells during C. albicans infection, suggesting that this antimicrobial peptide serves as a host defense substance also *in vivo*.

We also determined the effects of hGAPDH (2-32) on chemokine secretion and TLR4 expression of oral epithelial cells. In previous studies, it has been shown that neutrophil defensins increased the secretion of IL-8 by pneumocyte-like A549 and primary bronchial epithelial cells (van Wetering *et al.*, 2002; Sakamoto *et al.*, 2005). The increased epithelial expression of IL-8, GM-CSF, and TLR4 of infected epithelial cells induced by hGAPDH (2-32) in the present study supports the important role of human antimicrobial peptides for mucosal immunity by recruitment of activated leukocytes and lymphocytes to the site of oral infection. In addition, we demonstrated increased secretion of both cytokines and mRNA TLR4 expression by hGAPDH (2-32) also of uninfected epithelial cells compared with the PBS-treated controls. Recently, we demonstrated the important role of a PMN-mediated upregulation of epithelial TLR4 for protecting the oral mucosal surface from fungal invasion and cell injury (Weindl *et al.*, 2007). Upregulation of TLR4 also in the absence of PMNs suggests an immune conditioning mechanism by this peptide for host defense. An important role of hGAPDH (2-32)-induced immunomodulatory effects for tissue protection is suggested by the observation that epithelial damage in our infection experiments was significantly reduced in the presence of 5 $\mu g\,ml^{-1}$ hGAPDH (2-32), whereas the same concentration was not very effective in our killing assay.

In this study, we demonstrate that the antimicrobial peptide hGAPDH (2-32) is secreted by epithelial cells during mucosal candidiasis. The peptide has a high binding affinity to the fungal cell wall and may induce apoptosis after internalization. It partially antagonizes Sap activity, modulates the immune response, and causes profound changes in the yeast ultrastructure *in vitro*. The therapeutic relevance of these effects during *C. albicans* infection is confirmed by a strong protection against tissue damage in an established model of oral candidiasis. These data imply a protective role for the antimicrobial peptide hGAPDH (2-32) during experimental oral candidiasis.

In summary, antimicrobial agents not only acting against microorganisms in a classical way by fungicidal mechanisms but also interfering as apoptotic, antivirulence, and immunomodulating drugs are a promising approach to antimicrobial therapy.

MATERIALS AND METHODS

Experimental strategy to find antimicrobial peptides

To detect antimicrobial peptides, a peptide library from human placental tissue was prepared. The collected fractions were initially screened for growth-inhibitory activity against *E. coli* as described previously (Liepke *et al.*, 2003). A fraction demonstrating antibacterial activity was selected for further purification and analyzed until the active compound was identified. The synthesized peptide was used for further testing of antimicrobial activity against *C. albicans*. In addition, we tested the inhibitory activity against Saps and the therapeutic and immunomodulatory effects after infection of epithelial monolayers and in an *in vitro* model of oral candidiasis.

Preparation of the peptide library

Human placental tissue was obtained from healthy individuals in a maternity ward of a local hospital after informed written consent. The design of the work has been approved by the local ethics committee (ethics committee of Hannover Medical School), and the study was conducted according to the Declaration of Helsinki Principles. The tissue was processed immediately after delivery and extracted in ice-cold 0.5 M acetic acid containing 10 mM ascorbate and 0.5 mM EDTA. The extract was homogenized, filtered, and subsequently ultrafiltered as described previously (Liepke *et al.*, 2003). After cation-exchange chromatography, further subfractioning of each pH pool was carried out by reversed-phase chromatography (Liepke *et al.*, 2003). Using this procedure, 42 fractions of each pool were lyophilized and stored at −20 °C, designated the peptide library.

Purification of antimicrobial peptides from human placental tissue

Peptides were analyzed by electrospray mass spectrometry and by capillary zone electrophoresis. Amino-acid sequencing was performed by Edman degradation (Liepke *et al.*, 2003). Each purification step was monitored by the radial diffusion assay for detection of antimicrobial activity.

Peptide synthesis

The identified peptide hGAPDH (2-32) was synthesized using Fmoc solid-phase chemistry on a preloaded TentaGel F-PHB histidine TRT resin (Rapp Polymere, Tübingen, Germany) and was purified by reversed-phase HPLC (Vydac C18, 10 µm, 300Å, gradient: 10–70% B in 30 minutes, eluent A: 0.07 % trifluoroacetic acid/water, eluent B: 0.05% trifluoroacetic acid in acetonitrile/water 4:1, flow rate: 0.8 ml minute^{-1}, UV detection: 215/230 nm). Purity and identity of synthesized peptides were checked by analytical HPLC, mass spectrometry, and sequence analysis.

Microorganisms

For our antimicrobial experiments, we used *E. coli* ATCC 25922 and the clinical *C. albicans* isolate SC5314 (Gillum *et al.*, 1984). *C. albicans* strains 143 and 526 were isolated from feces, and strain 277 from tracheal secretions and provided by the Institute of Laboratory Medicine, Klinik am Eichert (Göppingen, Germany).

Antimicrobial assays

Flow cytometric antimicrobial assay measuring membrane depolarization of *E. coli* ATCC 25922 and fungi was carried out as described elsewhere (Nuding *et al.*, 2006; Schroeder *et al.*, 2011). Briefly, 1.5×10^6 cells of *E. coli*, and the *C. albicans* strains 143, 277, 526, and SC5314 were incubated in 1:6 diluted Schaedler broth at 37 °C with hGAPDH (2-32) in a final volume of 50 µl. The antimicrobial peptides hGAPDH (2-32), hBD-3 (Peptide Institute, Osaka, Japan), or LL-37 (Innovagen, Lund, Sweden) were dissolved in 0.01% acetic acid and added to bacterial and fungal suspensions at 10 µg ml^{-1}. To determine concentration dependence of microbial killing, hGAPDH (2-32) was added in concentrations of 5, 10, 50, 100, and 125 µg ml^{-1}. Microbial suspensions incubated with solvent (0.01% acetic acid) served as controls for viability. After 90 minutes, the suspensions were incubated for 10 minutes with 1 mg ml^{-1} of the membrane potential–sensitive dye DiBAC4(3) (Invitrogen, Karlsruhe, Germany). Suspensions were centrifuged for 10 minutes at 4,500 *g*, and the sediments were resuspended in 300 µl PBS. The percentage of depolarized fluorescent bacteria or fungi in suspension was determined by a FACSCalibur flow cytometer (BD, San Jose, CA) using the Cell Quest software (BD) for 10,000 events per sample.

Electron microscopy

Approximately 10^6 *C. albicans* SC5314 cells per ml were treated with hGAPDH (2-32) peptide in a concentration of 125 µg ml^{-1} and incubated for 24 hours at 37 °C. Treated and untreated yeast cells as a control were centrifuged, and the pellet was fixed in a PBS (0.05 M, pH 7.3) with 2.5% glutaraldehyde and 2% formaldehyde following standard methods. Postfixation was carried out with 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.3 at room temperature, and the specimens were embedded in glycid ether. Ultrathin sections, 20–30 nm thick, were mounted on uncoated copper grids and stained in 2% uranyl acetate for 3 minutes and examined using a Zeiss LIBRA transmission electron microscope (Oberkochen, Germany).

Sap inhibition assay

We used recombinant Sap1, Sap2, and Sap3 proteins expressed in *P. pastoris* to evaluate the inhibitory activity of hGAPDH (2-32) against single Sap isoenzymes. We incubated 0.25 µg of the indicated Sap protein with the increasing hGAPDH (2-32) peptide at nonpermissive pH 7.4 for 1 hour at room temperature in a 40-µl reaction volume. The Sap proteolytic reaction was then initiated by addition of substrate (BSA, 8 mg ml^{-1}) and pH shift to permissive pH 3.2 in a final volume of 100 µl (peptide/antibody concentration was calculated relative to the 100 µl final volume). The reaction was incubated for 30 minutes at 37 °C and stopped by addition of trichloroacetic acid. Trichloroacetic acid–soluble peptides were extracted and quantified using the bicinchoninic acid protein quantification kit from Thermo Scientific (Bonn, Germany) according to the manufacturer's indications. Error bars indicate range of duplicates. Each duplicate consists of three background-normalized measures.

Generation and affinity purification of the hGAPDH-peptide antibody

The antigenic peptide GKVKVGVNGFGRIGRLVTRAAFNSGKVDIVA hGAPDH (2-32) was synthesized on a multiple peptide synthesizer, Syro II (MultiSynTech, Witten, Germany), using the Fmoc-chemistry. The peptide was purified using reversed-phase HPLC to >90% purity, and the identity was confirmed using MALDI-MS-TOF analysis. The peptide was coupled to keyhole limpet hemocyanin using the glutardialdehyde method. The obtained antiserum was further purified by affinity chromatography on a CH-activated Sepharose 4B column (GE healthcare, Munich, Germany) containing the peptide immobilized via a stable peptide bond (Zaidi *et al.*, 2007). After concentration on a 20-kDa membrane, the resulting antibody was tested by ELISA on a microplate coated with GAPDH or the peptide hGAPDH (2-32). For control, the preimmune serum was used.

Analyses of apoptotic and necrotic markers

C. albicans cultures were grown overnight in YPD (yeast extract/peptone/dextrose) by shaking at 37 °C. A total of 1×10^7 cells in 250 µl PBS were treated with 5 µg ml^{-1} hGAPDH (2-32) at 37° for 60 minutes or with 50 mmol H_2O_2 as a positive control. After centrifugation, cells were protoplasted by digesting with SCE

(sorbitol/sodium-citrat/EDTA) buffer containing 100–500 U mg^{-1} Lyticase from *Arthrobacter luteus* (Sigma, Taufkirchen, Germany) and 2-mercaptoethanol (Roth, Karlsruhe, Germany) at 37 °C for 70 minutes. Samples were stained with FITC-labeled annexin-V (BD Pharmingen, Heidelberg, Germany) and propidium iodide (Molecular Probes, Darmstadt, Germany) to assess the externalization of phosphatidylserine and cell integrity. Cells were analyzed under a Leica TCS-SP/Leica DM RB confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with the Leica Confocal Software LCS (version 2.61).

Cytokine analysis

GM-CSF and IL-8 concentrations in the culture supernatants were determined using commercially available ELISA Kits (DuoSet, R&D Systems Minneapolis, MN).

RNA isolation and quantitative reverse transcriptase–PCR

Total RNA was extracted with NucleoSpin RNAII (Macherey-Nagel, Düren, Germany) and cDNA was synthesized using 1 μg total RNA with SuperScript III Reverse Transcriptase (Invitrogen). Amplification of cDNA was performed as described previously (Weindl *et al.*, 2007). All samples were run in duplicates and the mean threshold cycle (Ct) reading was used. Fold difference in gene expression was normalized to the housekeeping gene (Weindl *et al.*, 2007).

Models of oral candidiasis

For monolayer infection studies, the human buccal cell line TR146 was used. Cells were cultured in DMEM medium with 10% FCS and 0.1% gentamicin solution (50 mg ml^{-1}) at 37 °C in 5% CO_2. Infection studies (multiplicity of infection 0.1) were performed in antibiotic- and antimycotic-free culture medium. Epithelial monolayers were infected with *C. albicans* SC5314 in the presence and absence of 5, 10, 50, 100, and 125 μg ml^{-1} hGAPDH (2-32) for 12 and 24 hours. To evaluate tissue damage during infection, LDH analysis was performed (Weindl *et al.*, 2007).

The *in vitro* model of oral candidiasis was based on multilayer RHE. The mucosa equivalent and all culture media were prepared as described previously (Weindl *et al.*, 2007). Epithelial cultures were infected with 2×10^6 *C. albicans* SC5314 cells in 50 μl medium (3 g l^{-1} tryptic soy broth in 10 mM sodium phosphate buffer, pH 7.2) containing 5, 10, 50, 100, 125 μg ml^{-1} hGAPDH (2-32) or without peptide as a control. Infected cultures were incubated for 12 and 18 hours at 37 °C in a 100% humidified atmosphere containing 5% CO_2. To evaluate histological changes during infection, light microscopic studies were carried out as described previously (Weindl *et al.*, 2007). Histological changes of the mucosa were evaluated on the basis of 50 sections from five different sites for each infected epithelium.

For confocal microscopy, oral RHE was cryofixed in liquid nitrogen, and 5-μm sections were placed on Roti-Bond adhesion slides (Roth GmbH, Karlsruhe, Germany). Sections were fixed in periodate–lysine–paraformaldehyde (paraformaldehyde and lysine in PBS) for 2 minutes, followed by incubation with PBS for 5 minutes, PBS/BSA (0.1%) plus Tween 20 (0.1%) for 5 minutes, and PBS plus 10% donkey serum for 30 minutes at room temperature. Anti-hGAPDH (2-32) polyclonal rabbit antibodies (1:50) and human anti-*C. albicans* serum (1:60; Virion\Serion, Würzburg, Germany) were added for 60 minutes at room temperature. Sections were then incubated with donkey anti-rabbit–Dylight 549 (1:800; Dianova, Hamburg, Germany) and donkey anti-human–Cy5 (1:500; Dianova) for 60 minutes. All nuclei were stained with YOPRO (Invitrogen). All washing and antibody addition steps were performed with a combination of PBS, BSA, and Tween. The sections were analyzed with a confocal laser scanning microscope (Leica TCS SP; Leica Microsystems) at × 400 and × 250 magnification.

Statistics

All experiments were performed at least three times, if not otherwise indicated, and revealed comparable results. Results are presented as mean ± SD. Statistical significance was determined using the two-tailed paired Student's *t* test. A *P*-value of 0.05 or less was considered significant.

CONFLICT OF INTEREST

W-GF is CEO of IPF Pharmaceuticals GmbH and shareholder of Pharis Group. The remaining authors state no conflict of interest.

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